PRODUCTION IN MICE OF TOLERANCE TO THE TOXIC MANIFESTATIONS OF *CANDIDA ALBICANS*

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Received for publication March 22, 1962

ABSTRACT

HASENCLEVER, H. F. (National Institutes of Health, Bethesda, Md.) AND WILLIAM O. MITCHELL. Production in mice of tolerance to the toxic manifestations of *Candida albicans*. J. Bacteriol. **84**:402–409. 1962. — Lethal toxic manifestations of *Candida albicans* in mice were demonstrated by the intravenous injection of 10^7 viable yeast cells. Untreated control animals had an average survival time of 4.5 to 10 hr after challenge. No multiplication of *C. albicans* could be demonstrated in the tissues of untreated infected controls 12 hr after challenge. Mice infected ip with *C. albicans* 6 days before iv challenge survived for periods three to four times longer than controls. Mice previously infected with other fungi also demonstrated tolerance to the toxicity of *C. albicans*. *Coccidioides immitis* and *C. stellatoidea* were especially effective. High-titer rabbit antiserum to viable *C. albicans* yeast cells was not protective. Studies of total and differential peripheral leukocytes indicated that mice in the tolerant state demonstrated a slight granulocytosis. Direct evidence of the protective effect of the granulocytic response was not shown

The lethal toxic effect of viable *Candida albicans* yeast cells for mice has been demonstrated by the studies of Mourad and Friedman (1961*a*). These investigators found intact yeast cells most toxic but the cytoplasmic contents from sonically fractured cells possessed some activity. Our results with viable intact *C. albicans* cells are similar; we have not studied extensively cytoplasmic extracts obtained by sonic vibration of yeast cells. The contents of *C. albicans* obtained by disruption of yeast cells in a French press gave inconsistent and unreproducible results.

Previously, Salvin (1952) reported the presence of endotoxins in nonviable *C. albicans* cells, but adjuvants were necessary for demonstration of the lethal action. Roth and Murphy (1957) showed that antibiotics enhance the toxic activity of *C. albicans* in laboratory animals.

Investigations in our laboratory have shown that mice can be pretreated so that they are tolerant to the toxic effects of *C. albicans*. This paper describes the procedures that have been studied for the demonstration of tolerance to the toxicity of this yeast.

MATERIALS AND METHODS

General-purpose NIH or C.F.W. Swiss white mice (16 to 20 g), obtained from the Animal Production Section of the National Institutes of Health, have been used in this study. In the majority of experiments, female general-purpose mice were used, but no difference in results was noted when the other strains were used. On occasion, male mice of the same age and strains were utilized without any greater variation in reproducibility of observations than with female mice.

The strain of *C. albicans* used for this investigation was B311. It was isolated from a patient who died from generalized candidiasis. This strain possesses a high degree of virulence for mice; 10^5 yeast cells injected iv are lethal for 100% of the mice receiving this dose, 10^4 yeast cells kill 30 to 60% of the recipient animals, and 10^3 cells are lethal for about 5 to 15% of the mice. After injection of the aforementioned numbers of yeasts, death occurs in 5 to 30 days. For this study, we injected 10^7 yeast cells iv to show toxic activity. This number of cells killed most of the normal mice in 5 to 12 hr.

C. albicans cells for iv injection were obtained from stationary cultures in 2% glucose–1% Neopeptone broth incubated at 30 C for 48 to 72 hr. They were harvested and washed two to five times in 0.85% NaCl before injection. The number of cells in the suspensions was determined by direct count in a Levy hemocytometer and

by pour plates. Yeast cells for ip injection were harvested from 48-hr cultures grown on glucose-Neopeptone agar. Other fungi used in this study, with the exception of *Histoplasma capsulatum* and *Coccidioides immitis*, were cultured on the same medium. *H. capsulatum* was grown for 4 to 5 days at 37 C on blood-glucose-cystine agar. *C. immitis* spherules, provided by George Lones, were cultured at 37 C in Converse medium.

Pour-plate cultures of blood, spleen, liver, kidney, lung, and brain from infected mice (sacrificed at intervals indicated in Fig. 2 and 3) were made in 2% glucose-Trypticase Soy Agar. Three mice were killed at each interval. Blood was removed aseptically into a few drops of heparin, the mixture was serially diluted, and triplicate pour plates were made of the appropriate dilutions. The organs were removed separately, the like organs pooled (i.e., the spleens from three mice, livers from three mice, etc.), ground in sterile mortars with pestles, serially diluted, and plated. The skins and gastrointestinal tracts were removed; the carcasses were homogenized in a Waring Blendor, diluted, and plated in agar containing 50 μg of chloramphenicol/ml. The plates were observed for *C. albicans* colonies after 48 hr of incubation at 30 C.

Preliminary studies indicated that an ip infection with 10^7 *C. albicans* B311 cells, to which mice are quite resistant, would protect most of the animals against the toxic effect of subsequent iv injection of this organism, but the mice still died of overwhelming kidney infection due to the iv challenge dose. Subsequently, experiments were done with other fungi to test the specificity of this observation. The effect of ip infection with *C. tropicalis*, *C. stellatoidea*, *C. guilliermondii*, *C. parapsilosis*, *Torulopsis glabrata*, *H. capsulatum*, *C. immitis*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* was studied. Nonviable suspensions of *C. albicans* cells and *C. immitis* spherule particles were also investigated.

After iv challenge, mice were observed for deaths hourly up to 12 hr after injection. This was the period during which the majority of control mice would succumb. Some of the tolerant mice in each experiment died at this time but most survived considerably longer. After this initial period, the mice were observed at intervals of 3 to 8 hr.

The average survival time was calculated by determining the total survival hours of a group of mice, and dividing by the number of animals in a group. If it was observed that ten mice died between the 6th and 7th hour after challenge, it was considered that 6.5 hr was the average survival time of each animal, and therefore the group survived a total of 65 mouse hours. Likewise, if four mice died between the 48th and 51st hours, 49.5 hr was considered the average death time and these mice were calculated to have survived a total of 198 mouse hours. This correction procedure was applied to observations of all groups of mice throughout the study.

Statistical analysis, on some of the data obtained in these experiments, was done employing a one-sided Kolmogorov-Smirnoff test. (The authors are indebted to David W. Alling for these analyses.)

Passive protection tests with antiserum to viable *C. albicans* yeast cells were also conducted. To produce such an antiserum, strain 857 (with a low degree of pathogenicity for rabbits) was used. This strain demonstrated less toxic activity than the study strain, but 2×10^7 cells administered to mice iv gave the characteristic lethal manifestations. The antiserum had an agglutinating titer of 1:5,120; it was diluted 1:5, and 0.5 ml was injected ip into mice 2 hr before challenge. Control groups of mice receiving similar amounts of normal rabbit serum or sterile saline were included.

Peripheral blood studies were done on mice in the pretolerant and tolerant stages. Individual determinations were conducted on animals, in groups of five, before and 6 days after injection of agents that induced tolerance. Blood was drawn from the periorbital plexus into heparin-rinsed capillary pipettes. Standard methods for total and differential leukocyte counts were employed.

RESULTS AND DISCUSSION

The results from mice made tolerant by ip preinfection and from untreated controls, challenged with 10^7 *C. albicans* cells iv, are shown in Fig. 1. Of the controls, 80% had died 12 hr after injection, whereas only 10% of the tolerant mice had succumbed. The average survivor time of the preinfected mice was four times that of the controls. The data in Table 1 are presented in this manner to illustrate the range of mortality observations. While the average survivor times

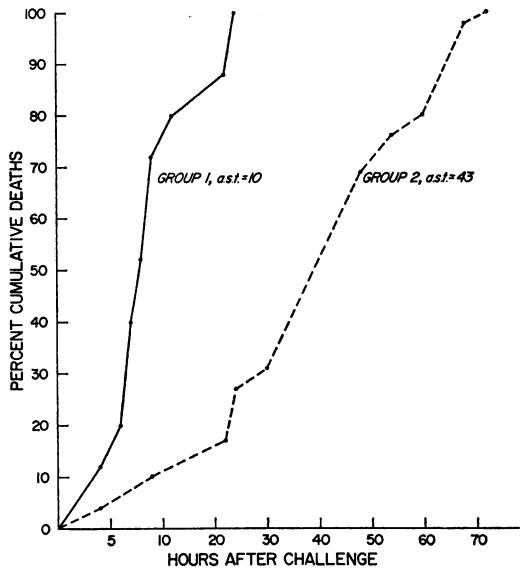

FIG. 1. *Tolerance produced by ip preinfection with Candida albicans;* a.s.t. = *average survival time. Group 1, infected ip with* 10^7 *C. albicans cells 6 days before iv challenge, 29 mice. Group 2, controls, iv challenge only, 25 mice.*

TABLE 1. *Statistical significance of differences in survival when tested with a one-sided Kolmogorov-Smirnoff test*

Species	Maximal difference in survival between treated and controls	Significance level P
Candida albicans	0.852	.01
C. tropicalis	0.367	.01–.05
C. stellatoidea	0.800	.01
C. guilliermondii	0.334	.01–.05
C. parapsilosis	0.334	.01–.05
Torulopsis glabrata	0.367	.01–.05

may be helpful for comparative purposes, they are sometimes misleading and their meaning and interpretation are clarified by the per cent of cumulative deaths at the stated times.

The results in Fig. 1 immediately raised the question of multiplication of *C. albicans* within the preinfected and control groups of mice. The majority of control mice died within 12 hr, and with the large inoculum used, it was necessary to demonstrate whether the mice died of overwhelming infection or toxicity of the pathogen. If little or no growth of *C. albicans* could be detected at 12 hr after challenge, it would be good evidence that the mice had died of toxins from the yeast. There was an obvious difference in survival times between preinfected and control mice, and it was necessary to determine whether the preinfected animals suppressed the growth of *C. albicans* to a greater extent.

Figure 2 shows the multiplication of *C. albicans* in the control mice. No discernible growth could be detected 12 hr after challenge (when the majority of mice had died), but at 24 hr a considerable amount of multiplication had occurred in the kidneys. The mice used in this experiment were taken from the control group appearing in Fig. 1. Since these mice were sacrificed, the results, particularly 6 hr after challenge and later, were obtained from surviving and possibly resistant animals. This was especially true of the 24-hr plating for which only two mice still remained. The data indicate, within the limitations of the methods employed, that 12 hr after challenge, when 80% of the mice have died, no detectable multiplication of *C. albicans* can be shown. Evidence suggests that death was due to toxicity rather than overwhelming infection.

The results of the multiplication of *C. albicans* in preinfected mice are shown in Fig. 3. Although these animals were injected ip with 10^7 viable *C. albicans* cells 6 days before the iv challenge, the initial infecting dose apparently has been

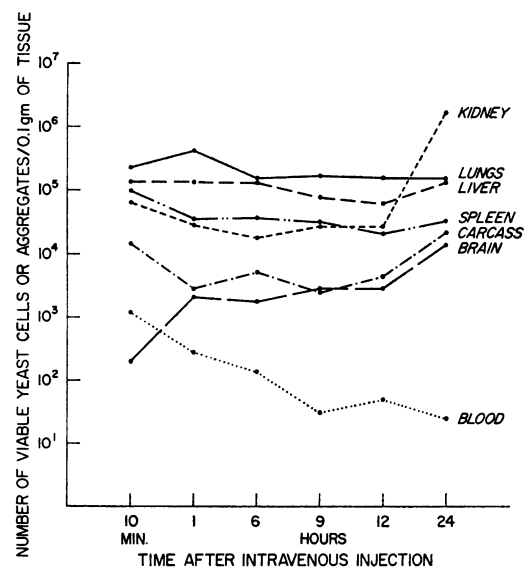

FIG. 2. *In vivo growth of Candida albicans in control mice.*

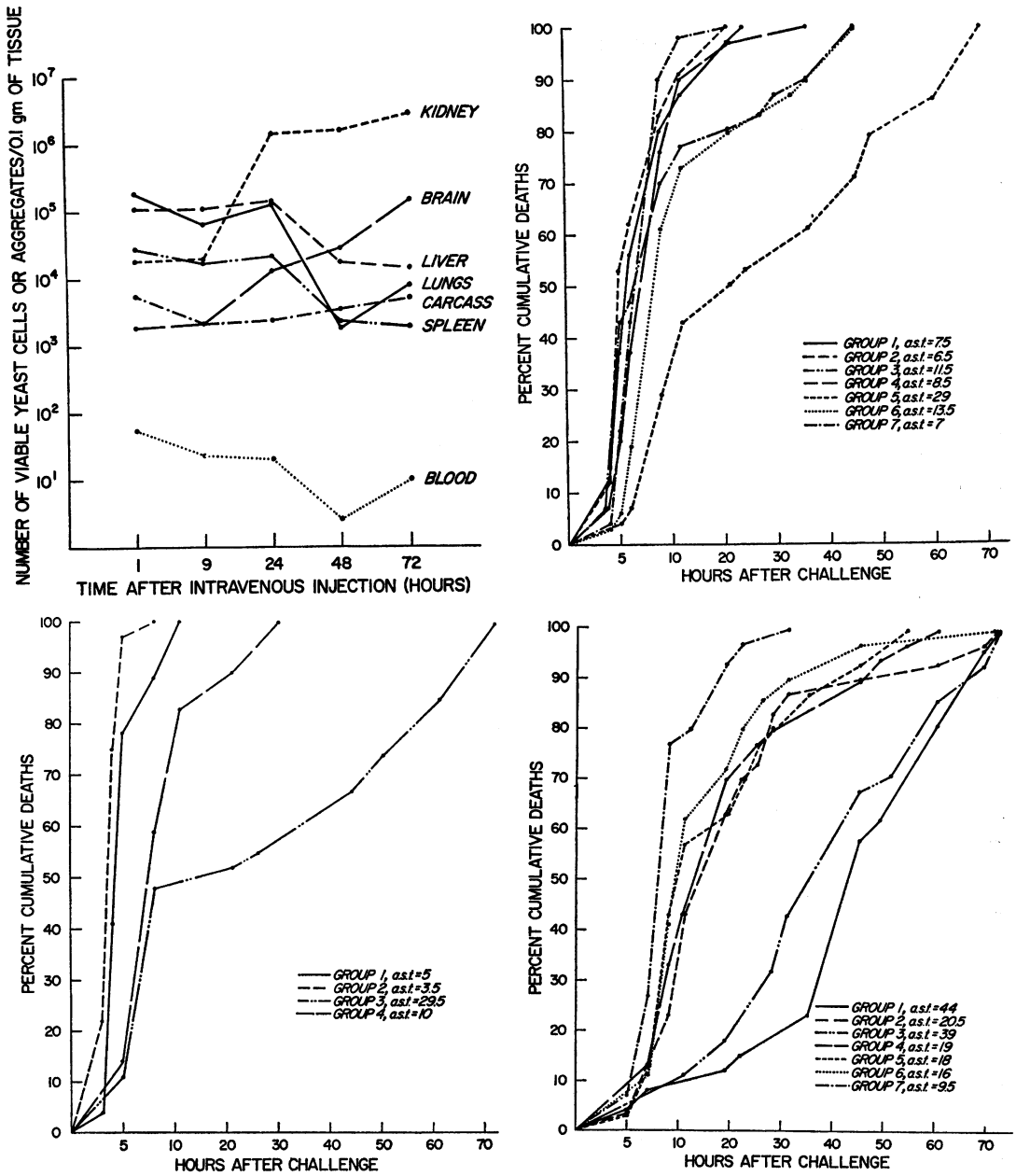

FIG. 3. (top left) *In vivo growth of Candida albicans in tolerant mice.*

FIG. 4. (top right) *Tolerance produced by heat-killed Candida albicans cells. Group 1, 5 × 10⁷ nonviable cells (1.4 mg dry wt) iv 6 days before iv challenge, 30 mice. Group 2, 10⁷ nonviable cells (0.28 mg dry wt) iv 6 days before iv challenge, 32 mice. Group 3, 5 × 10⁷ nonviable cells (1.4 mg dry wt) ip 6 days before iv challenge, 30 mice. Group 4, 10⁷ nonviable cells (0.28 mg dry wt) ip 6 days before iv challenge, 30 mice. Group 5, 10⁷ viable cells ip 6 days before iv challenge, 28 mice. Group 6, 10⁶ viable cells ip 6 days before iv challenge, 31 mice. Group 7, controls, iv challenge only, 49 mice.*

FIG. 5. (bottom left) *Effect on tolerant mice when injected with a larger challenge dose. Group 1, 10⁷ viable cells ip 6 days before iv challenge with 5 × 10⁷ cells, 27 mice. Group 2, controls, challenged iv with 5 × 10⁷ cells, 32 mice. Group 3, 10⁷ viable cells ip 6 days before iv challenge with 10⁷ cells, 27 mice. Group 4, controls, challenged iv with 10⁷ cells, 29 mice.*

FIG. 6. (bottom right) *Tolerance produced by preinfection with other species of Candida and Torulopsis glabrata. Group 1, 10⁷ C. albicans cells ip 6 days before iv challenge, 26 mice. Group 2, 10⁷ C. tropicalis cells ip 6 days before iv challenge, 30 mice. Group 3, 10⁷ C. stellatoidea cells ip 6 days before iv challenge, 28 mice. Group 4, 5 × 10⁷ C. guilliermondii cells ip 6 days before iv challenge, 30 mice. Group 5, 5 × 10⁷ C. parapsilosis cells ip 6 days before iv challenge, 30 mice. Group 6, 5 × 10⁷ T. glabrata cells ip 6 days before iv challenge, 29 mice. Group 7, controls, iv challenge only, 30 mice.*

destroyed by the peritoneal host defenses. The total number of viable *C. albicans* cells recovered from the tissues of these mice was about the same as for the control mice. The results, obtained at 24 hr (the time at which all control mice were dead or in the terminal stages of disease), showed that over 70% of the preinfected, tolerant mice were still living. The tissue levels of viable *C. albicans* cells or aggregates at 9 hr in both groups of mice were quite similar, although minor differences were detected in the livers and lungs. At 24 hr, the tissue levels were again quite comparable, except that carcasses of the control mice showed about a 1 log higher level than the tolerant animals. Although most of the preinfected mice survived the period of acute toxic manifestations (6 to 12 hr after injection), it is apparent (Fig. 2) that they finally died of an overwhelming infection. The data, obtained by these methods, indicate that the tolerant mice did not appear to be able to suppress the tissue growth of *C. albicans* significantly more than did the control mice.

Figure 4 shows the effectiveness of heat-killed *C. albicans* cells in producing, in mice, the state of tolerance. Two different doses, 5×10^7 or 10^7 yeast cells per mouse, were used, and they were administered by two different routes. Groups of mice receiving 10^7 or 10^6 viable yeast cells ip were included as infected controls. As indicated in Fig. 4, groups 1, 2, and 4, which received the nonviable suspensions, demonstrated little tolerance. Group 3 (5×10^7 nonviable cells ip) and group 6 (10^6 viable cells ip) showed some tolerance, and group 5 (10^7 viable cells ip) possessed the usual level of tolerance. The results of this experiment illustrate how the calculated average survival time is somewhat misleading. In this experiment, 50% of the tolerant mice had died 21 hr after iv challenge, although 100% of the controls had succumbed. The extended average survival time of the tolerant groups was due primarily to the 13 animals (47%) that lived beyond 24 hr.

In addition to testing the amount of tolerance produced in mice with nonviable *C. albicans* cells, the data in Fig. 4, especially results from groups 1 and 2, are helpful in eliminating the possibility that deaths in the control animals were due to embolic phenomena. Mice in groups 1 and 2 received iv 5×10^7 and 10^7 heat-killed yeast cells, respectively, without showing any gross effects. The question of deaths, due to emboli in animals injected iv with large numbers of microorganisms the size of yeast cells, is raised frequently. Since the challenge dose for all groups was the same, it seems most unlikely that group 5 would be more resistant to emboli than the other groups. It is possible that after 6 to 12 hr in the host, the yeast cells started to germinate and then the parent cell and its bud or germination tube became a focus for the formation of an embolus. The same process, however, should have occurred in the preinfected group.

Results of an experiment designed to test the effectiveness of the preinfection tolerance with a larger challenge dose are shown in Fig. 5. As indicated, mice in group 1 survived slightly longer than those in group 2, the corresponding controls, while mice in group 3 survived about three times longer than those in group 4. It is quite obvious that sufficient tolerance was not produced to extend the average survival time significantly for mice injected with the larger challenge dose.

The tolerance of mice preinfected with antigenically related yeasts is shown in Fig. 6. In this experiment, the homologous strain appeared to be most effective in eliciting the altered host state, and was followed closely by *C. stellatoidea*. *C. tropicalis*, although more closely related antigenically to B311, a group A strain, was less effective than *C. stellatoidea*. Preinfection with the other species of yeasts included in this experiment did not confer, to the mice, tolerance of the same level as *C. albicans* or *C. stellatoidea*.

An analysis of the statistical significance of the data in Fig. 6 is shown in Table 1. Preinfection with any of the yeasts included in this experiment conferred tolerance that was statistically significant.

To test further the production of tolerance, infections in mice with other pathogenic fungi were studied. Those results are shown in Fig. 7. It is quite apparent that infections with all the fungi investigated increased the resistance of mice to the toxicity of *C. albicans*. Protection conferred by *C. immitis* infection was especially effective, being comparable or superior to that produced by *C. albicans*. The data shown in Fig. 6 and 7 indicate the nonspecific nature of the tolerance phenomenon.

Since infection with *C. immitis* was quite effective in producing the tolerant state, studies

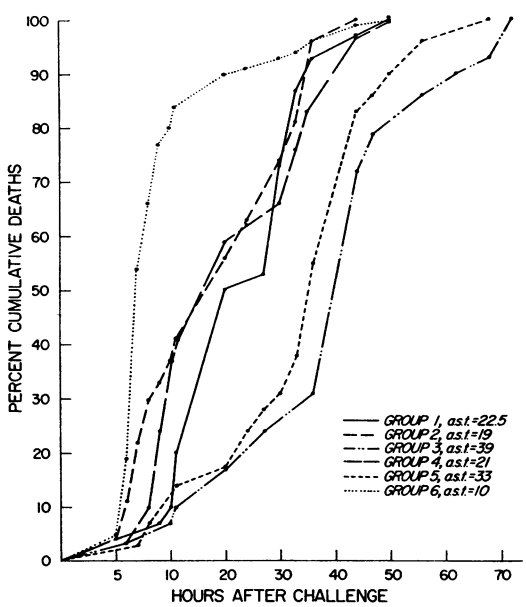

FIG. 7

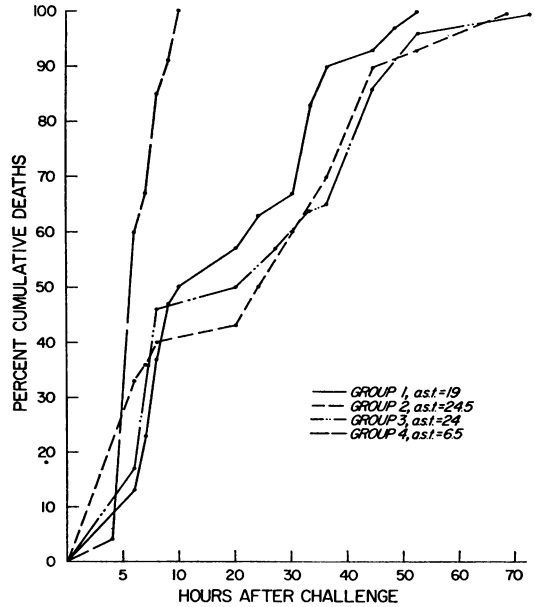

FIG. 8

FIG. 7. *Tolerance produced by other pathogenic fungi. Group 1, 10^6 Histoplasma capsulatum cells ip 6 days before iv challenge, 30 mice. Group 2, 10^7 Cryptococcus neoformans cells ip 6 days before iv challenge, 27 mice. Group 3, 4 × 10^4 Coccidioides immitis viable spherule units ip 6 days before iv challenge, 29 mice. Group 4, 10^7 Aspergillus fumigatus conidiospores ip 6 days before iv challenge, 29 mice. Group 5, 10^7 Candida albicans cells ip 6 days before iv challenge, 30 mice. Group 6, controls, iv challenge only, 70 mice. Ten mice from each of the infected groups were not challenged and were held as infected controls. No animal in these control groups died during the experimental period. These animals were not included in the above groups.*

FIG. 8. *Tolerance produced by viable and nonviable Coccidioides immitis spherules. Group 1, 4 × 10^4 viable spherule units ip 6 days before iv challenge, 30 mice. Group 2, 1 mg formalin-killed spherule fragments (exposed to 25,000 psi in French pressure cell) ip 6 days before iv challenge, 30 mice. Group 3, 10^7 viable Candida albicans cells ip 6 days before iv challenge, 28 mice. Group 4, controls, challenged only, 54 mice.*

with formalin-killed, broken *C. immitis* spherules were done. Those results are shown in Fig. 8. It is quite apparent that the nonviable spherules were quite effective. Although mice in group 3 (Fig. 4) received approximately the same unit of dried *C. albicans* cells by the same route, they did not demonstrate the same amount of tolerance as the mice injected with nonviable *C. immitis* spherules.

Passive protection of mice with antisera to *A. fumigatus* toxins has been reported (Henrici, 1939; Tilden, Williamson, and Koenig, 1960). It was reasonable to assume that *C. albicans* antisera would also be protective. The results of attempts to show passive protection with high-titer antiserum to viable *C. albicans* cells are shown in Fig. 9. It is obvious that this antiserum had no protective action.

A possible mechanism in the development of tolerance to the toxins of *C. albicans* is the alteration of the reticuloendothelial system or the circulating leukocytes. To test this possibility, especially changes in the circulating white blood cells, 15 mice were bled from the periorbital plexus, and total and differential leukocyte counts were made. Five mice were then injected ip with 10^7 viable *C. albicans* cells in saline, five were injected ip with 10^7 heat-killed *C. albicans* cells in saline, and five received ip 1 mg (dry wt) of nonviable broken *C. immitis* spherules suspended in saline; 6 days later, the mice were again bled from the periorbital plexus and total and differential counts again were made. The results before and after treatment with the tolerance-inducing agents and the controls (10^7 nonviable *C. albicans* cells) are shown in Table 2. All methods of treatment produced a measurable leukocytosis, although the mice receiving the

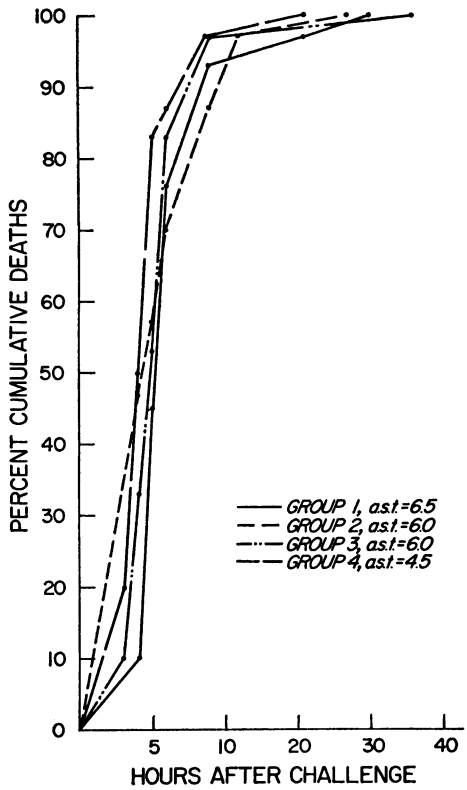

FIG. 9. *Effect of antiserum on toxicity of Candida albicans. Group 1, 0.5 ml of 1:5 antiserum, 2 hr before iv challenge, 29 mice. Group 2, 0.5 ml of 1:5 normal rabbit serum, 2 hr before iv challenge, 30 mice. Group 3, 0.5 ml of physiological saline, 2 hr before iv challenge, 30 mice. Group 4, controls, iv challenge only, 30 mice.*

heat-killed cells showed less than the other two. A neutrophilic response was observed in mice receiving viable *C. albicans* cells and the *C. immitis* preparation, but to a lesser extent with heat-killed *C. albicans* cells. The differences, however, are not very impressive.

These results indicate that there is some alteration, qualitatively and quantitatively, of the circulating leukocytes in the tolerant animals, but direct evidence of the protective role is not apparent. Louria and Browne (1960) showed that the enhancement of candidiasis by cortisone may be due, in part, to suppression of polymorphonuclear leukocyte activity. Sheldon and Bauer (1959), studying acute alloxan diabetus in rabbits, found suppressed numbers of polymorphonuclear leukocytes and suggested that this was an important factor in their increased susceptibility to mucormycosis. In the studies reported here, there was no apparent evidence that the tolerant mice could suppress the growth, in vivo, of *C. albicans* any more effectively than could the control mice. It did appear, however, that the tolerant animals cleared *C. albicans* cells from the bloodstream faster than did the controls. This process would be most likely a function of endothelial phagocytes rather than circulating ones. The interpretation that the concomitant neutrophilic response is associated with the protection demonstrated by tolerant mice can only be speculative.

Microscopy of histological sections made from the tissues of control mice in the acute toxic stages before death did not show any changes.

TABLE 2. *Total and differential leukocyte counts before and after injection with tolerance-inducing and nontolerance-inducing agents**

Agent	Preinjection						6 Days postinjection					
	Total WBC per mm³ blood†	Differential count (%)					Total WBC per mm³ blood	Differential count (%)				
		L	N	M	E	B		L	N	M	E	B
B311 viable	2,960–5,600	54–70	26–43	1–4	0–1	0–1	2,800–6,000	28–64	33–69	1–3	0–1	0–1
	3,890	60	36	3	0.6	0.2	4,800	50	47	2	1	1
B311 nonviable	2,560–6,300	44–63	34–54	0–6	0–1	0–0	2,800–7,520	40–70	26–66	3–4	0–1	0–1
	3,960	56	41	2	1	0	4,590	52	46	4	1	1
C. immitis nonviable	2,800–5,240	52–71	29–42	0–6	0–2	0–1	4,280–10,800	43–48	49–57	0–4	0–0	0–0
	4,030	61	33	4	0.6	0.2	6,110	46	53	2	0	0

* Abbreviations: L, lymphocytes; N, neutrophiles; M, monocytes; E, eosinophiles; B, basophiles; WBC, white blood cells.

† Range and average.

Death apparently occurs before any observable pathological alterations take place.

The observation of tolerance to the toxic effects of *C. albicans* represents an interesting immunological phenomenon. The mechanism is apparently unrelated to circulating antibodies and is probably associated with cellular alterations in the host. Although a granulocytosis can be demonstrated in the tolerant mice, this may be a corollary process. The nonspecific nature of the tolerant state suggests, however, that the mechanism resides at the cellular level. Studies are in progress in this laboratory to test the role of bacterial endotoxins and Freund's adjuvant in inducing tolerance to the toxins of *C. albicans*.

Investigations in this laboratory of the lethal activity of cell-free cytoplasmic components from ruptured viable *C. albicans* yeast cells have been inconsistent and unreproducible. Our method of fracture has been to treat cell suspensions to a pressure of 25,000 psi in a French pressure cell. Double exposure of a suspension to this process results in 75 to 90% breakage of the cells. Mourad and Friedman (1961*a*) reported more consistency with cell-free components from sonically ruptured cells. Because of greater reproducibility, we have used intact viable yeast cells for these studies.

It is quite apparent from the data shown in the figures that some variation of results occurred. The average survival time of the untreated control animals in the various experiments ranged from 4.5 to 10 hr; the tolerant mice varied from 24 to 44 hr. The average survival time of the tolerant animals from all experiments was three to four times that of the control mice, and was directly proportional (i.e., the average survival time of the controls was lower, so was that of the tolerant mice).

These experiments do not provide evidence as to whether tolerance to the acute toxic action of *C. albicans* is identical with resistance to chronic candidiasis. Mourad and Friedman (1961*b*) reported increased resistance in mice to chronic candidiasis after injection with viable or sonically disrupted *C. albicans* cells. The inability of antigenically related yeasts, such as *T. glabrata* and the *Candida* species studied (compared with the ability of *C. immitis*, an antigenically unrelated species), to confer tolerance raises important basic immunological questions. Hedgecock (1961) reported evidence of some increased resistance to tuberculosis in animals immunized with several different pathogenic fungi. Louria (1960) found that bacterial endotoxins enhanced resistance to experimental cryptococcosis about as much as cryptococcal vaccines. It appears that nonspecific resistance plays an important role in systemic fungus infections.

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